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Originals

A New Dibenzofuran, Isostrepsilic Acid, Produced by Cultured Mycobiont of the Lichenized Ascomycete *Usnea orientalis*

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Two dibenzofurans, isostrepsilic acid and hypostrepsilic acid, were produced in large amounts by the mycobiont of *Usnea orientalis* cultured on malt-yeast extract medium containing sugar alcohols. These substances were not detected in the natural lichen thalli growing in the field which normally produces usnic acid and salazinic acid. Isostrepsilic acid is a new lichen substance which has been determined to be 3,7-dihydroxy-9-hydroxymethyl-1-methyldibenzofuran-2-carboxylic acid.

Introduction

Thalli of *Usnea confusa* Asah. subsp. *kitamiensis* (Asah.) Asah. and *U. orientalis* Asah. can be routinely induced from their cultured tissue *in vitro*, and the induced thalli are similar to those of natural ones morphologically as well as chemically. However, the secondary metabolites produced by the mycobionts of the two species were different from those of the cultured thalli containing photobionts (Kon et al. 1993a).

When the mycobiont was cultured on a malt-yeast extract medium containing sugar alcohols, we found an accelerated growth of the mycobiont and the development of thalli (Kon et al. 1993b). Simultaneously, a large quantity of extracellulary compounds were produced. These compounds were subsequently isolated and their structural elucidation is described in this paper.

Materials and Methods

Culture conditions

Thalli of *Usnea orientalis* were collected on Mt. Kuju, Ooita Prefecture, in August, 1989. The mycobiont was obtained by the following procedures. Growing tissue was obtained from thalli following the technique described by Yamamoto et al. (1985). The tissue was aseptically placed into a sterilized mortar and then homogenized with sterilized distilled water. The homogenate was cultured on a medium composed of 2% malt extract, 0.2% yeast extract and 2% agar. After one month, mycobiont colonies could be separated and transferred on a plate. The mycobiont tissues thus isolated were used for the present study.

The following materials were purchased from sources listed in parentheses: malt extract (Difco); yeast extract (Difco); mannitol (Wako Pure Chemical); sorbitol (Wako Pure

Chemical); Wako-gel G200 (Wako Pure Chemical). Other chemicals were of the reagent grade.

The mycobiont was inoculated in test tubes containing malt-yeast extract medium containing 2% agar or on the same medium containing 2% mannitol and 2% sorbitol at pH 5.8. The mycobionts were cultured at 18°C in dark for 10 weeks. For comparison, the cultured mycobiont was mixed with its natural photobiont (identified as *Trebouxia irregularis* Hildreth & Ahmadjian by Dr. T. Nakano, pers. comm.). This resynthesized lichen was then cultured on a malt-yeast extract medium under the light (C. 13 μmol/m^2/s).

Isolation of the dibenzofurans

After 10 week culture, the mycelia of the mycobiont were separated from the agar and dried at 50°C for 24 hours. The dried mycelia (20 g) was then extracted overnight with 5 volumes of cold acetone. The supernatant thus obtained was concentrated to dryness under the reduced pressure. The residue was dissolved in a small amount of solvent A (methanol:chloroform=15:35,v/v) and subjected to column chromatography (Wakogel C-200, 2.0×20cm) using solvent A as elutant. The dibenzofurans so obtained were characterized by the standardized thin-layer chromatography method (Culberson and Elix 1990). The respective compounds obtained yielded a single spot on the chromatography.

Identification and quantification of the compounds

The TLC and HPLC methods were used for the identification of the compounds produced by mycobiont with or without photobiont. Mixtures of the compounds were analyzed by high performance liquid chromatography (HPLC) using a JASKO HPLC system as follows.

Dried and powdered mycelia of mycobiont or mycobiont associated with photobiont (100 mg) were extracted with 0.5 ml of acetone at room temperature for 15 min. The extract was then filtered through chromatodisk 13N (TOSO). The column was (4.6 × 150 mm) contained Finepack SIL $C_{18}S$ (Nippon Bunko 5 μm). The compounds obtained were eluted isocratically with methanol/water/H_3PO_4 (80:20:0.2, v/v). The flow rate was maintained at 1.0 ml/min (pressure, 50 kg/cm^2) and the elution of compounds was monitored by absorbance at 230 or 240 nm. The retention times of each of the secondary substances in sample were checked by using authentic compounds under the identical conditions. The amount of the compounds was determined from their peak areas by comparison with appropriate standards.

Table 1. Chromatographic (TLC) data

Compounds	Rf (×100) A	B	C
Salazinic acid	14	3	3
Usnic acid	57	68	57
Hypostrepsilic acid	31	41	19
Isostrepsilic acid	10	11	3
Norstictic acid (standard)	40	19	19
Atranorin (standard)	58	73	60

Table 2. High performance liquid chromatographic data

Compounds	Retention time (min)
Salazinic acid	2.20
Isostrepsilic acid	2.33
Hypostrepsilic acid	3.27
Norstictic acid	4.49
Usnic acid	19.03

Results and Discussion

Under natural conditions, *Usnea orientalis* produces both usnic acid (1.32%) and salazinic acid (2.16%, Table 3, lane a). The former substance is produced in the cortex, while the latter is a medullary product. When the mycobiont of *U. orientalis* was cultured on a malt-yeast extract medium it produced small amounts of usnic and salazinic acids (Table 3, lane b). Further, when this mycobiont was cultured with its natural photobiont, larger amounts of usnic and salazinic acids were produced (Table 3, lane c).

When the mycobiont was cultured on a malt-yeast extract medium containing sugar alcohols it produced a large quantity of extracellular dibenzofurans, which were not detected in the natural *U. orientalis* or the mycobiont with or without photobiont cultured on malt-yeast extract medium (Table 3, lane d).

The Rf values and retention times of the dibenzofurans I and II are compared with those of authentic compounds in Tables 1 and 2. On TLC plates the spots due to these compounds were pinkish gray in color after spraying with 10% sulphuric acid and heated at 110°C for 10 min.

Table 3. Secondary metabolites produced by natural *Usnea orientalis* and the mycobiont with or without its photobiont under various conditions

	Samples[1)]			
	A	B	C	D
Usnic acid	13.2[2)]	0.12	0.64	ND[3)]
Salazinic acid	21.6	0.04	1.61	ND
Hypostrepsilic acid	ND	ND	ND	4.91
Isostrepsilic acid	ND	ND	ND	1.46

[1)]A: Natural *Usnea orientalis* Asah. B: Mycobiont cultured on malt-yeast extract medium. C: Mycobiont with its natural photobiont on malt-yeast extract medium. D: Mycobiont cultured on malt-yeast extract medium containing 2% mannitol and 2% sorbitol.
[2)]mg/g.dr.wt
[3)]ND=not detected

The molecular formulae of the compounds I and II were established as $C_{15}H_{12}O_5$ and $C_{15}H_{12}O_6$, respectively, based on the corresponding high resolution mass spectral and/or microanalytical data. The respective chemical structures were deduced from the corresponding ^1H NMR and UV spectral data.

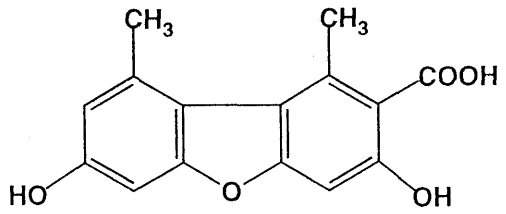
Compound I was identified to be 3,7-dihydroxy-1,9-dimethyldibenzofuran (Fig. 1A), a compound previously reported as a hypostrepsilic acid from the cultured mycobiont of *Evernia esorediosa* (Müll. Arg.) DuRietz under the osmotically stressed condition (Miyagawa et al. 1993), or as a norascomatic acid from *Bunodophoron patagonicum* (Dodge) Wedin (Elix et al. 1994).

With both the *Usnea* and *Evernia* mycobionts, hypostrepsilic acid was produced when the mycobionts were cultured on a malt-yeast extract medium containing sugar or sugar alcohol.

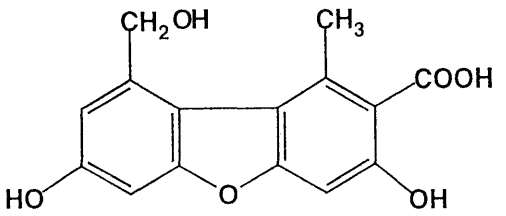
The spectroscopic and microanalytical evidence established that isostrepsilic acid was corresponding 9-hydroxymethyl derivative of compound I, i.e. 3,7-dihydroxy-9-hydroxymethyl-1-methyldibenzofuran-2-carboxylic acid (Fig. 1B). This compound has not previously been reported from lichens or mycobionts.

As hypostrepsilic acid and isostrepsilic acid were only produced when the malt-yeast extract medium contained additional sugar alcohol, the alternative biosynthetic pathway may be a consequence of the ensuing high osmotic pressure. Even so, it would be thought that the production of these compounds probably due to the combined effects of the high osmotic pressure of medium and the nutritional conditions.

Thus, it can be presumed that biosynthetic



A



B

Fig. 1. A. Chemical structure of hypostrepsilic acid [3,7-dihydroxy-1,9-dimethyldibezofuran-2-carboxylic acid]. B. Chemical structure of isostrepsilic acid [3,7-dihydroxy-9-hydroxymethyl-1-methyldibezofuran-2-carboxylic acid].

pathways to secondary products are blocked on route, and the alternative pathways may be open to produce different compounds not found in natural lichen thalli, especailly when a mycobiont is cultured on a medium enriched with sugar alcohol.

Hypostrepsilic Acid

The crude hypostrepsilic acid crystallized from acetone-light petroleum formed cream coloured microcrystals, m.p. 228–230°C (227–229°C), alone or admixed with a synthetic sample. (Found: C, 66.0; H, 4.6%; mol. wt. 272.0684. Calc. for $C_{15}H_{12}O_5$; C, 66.2; H, 4.4%; mol. wt. 272.0685). UVλ_{max} (EtOH) nm (logε): 309 (4.2), 257 (4.5), 241 (4.65). ^{1}H NMR (CD_3SOCD_3) δ 2.72 (3H, *s*, 9-Me); 3.03 (3H, *s*, 1-Me); 6.55, 6.75 (each 1H, *d*, J=2.2 Hz, H-6, H-8); 6.60 (1H, *s*, H-4); 9.74 (1H, *bs*, OH). EIMS (70eV) *m/z* 273 (5%), 272 (M, 25), 255 (18), 254 (100), 229 (7), 228 (60), 227 (23), 199 (5), 198 (22).

Isostrepsilic Acid

The crude isostrepsilic acid crystallized from 10% aqueous acetone formed cream coloured microcrystals, m.p. 210–220°C dec. (Found: C, 62.2; H, 3.2%. $C_{15}H_{12}O_6$ requires C, 62.5; H, 3.2%). UVλ_{max} (EtOH) nm (logε): 309 (4.3), 259 (4.6), 241 (4.7). ^{1}H NMR (CD_3SOCD_3) δ 3.01 (3H, *s*, 1-Me), 4.88 (2H, *d*, J=3.7 Hz, CH_2); 5.80 (1H, *t*, J=*s*, 7 Hz, 9-OH); 6.60 (1H, *s*, H-4); 6.77, 6.89 (each 1H, *d*, J=2.2 Hz, H-6, H-8); 9.65 (1H, *bs*, OH). EIMS (70 eV) *m/z* 270 (12), 241 (22), 228 (23), 227 (26), 213 (62), 199 (35), 185 (100).

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近　芳明，岩科　司，柏谷博之，J. H. ワードロー，J. A. エリックス：**地衣類ハナサルオガセ（*Usnea orientalis*）培養菌の産生する新規ジベンゾフラン**

ハナサルオガセ（*Usnea orientalis*）は試験管内での地衣体の再分化が容易な種である．地衣体由来の菌に対する培地に含まれる糖アルコールは，成長だけでなく，菌組織表面における突起上の構造物の形成を促進するものと認められている．菌の二次代謝産物の産生における糖アルコールの効果を調べたところ，天然には検出されない2種のジベンゾフランが検出された．その一つは3, 7-dihydroxy-1, 9-dimethyldibenzofuran-2-carboxylic acidと認められ，ヤマヒコノリ（*Evernia esorediosa*）の培養菌から報告されているhypostrepsilic acidと同一のものであった．しかし，他の成分は新規物質で3, 7-dihydroxy-9-hydroxymethyl-1-methyldibenzofuran-2-carboxylic acid (isostrepsilic acid）と構造決定された．